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Identification of ATP-Dependent Phosphofructokinase as a Regulatory Step in the Glycolytic Pathway of the Actinomycete *Streptomyces coelicolor* A3(2)

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The ATP-dependent phosphofructokinase (ATP-PFK) of *Streptomyces coelicolor* A3(2) was purified to homogeneity (1,600-fold) and characterized (110 kDa, with a single type of subunit of 40 kDa); it is allosterically inhibited by phosphoenolpyruvate. Cloning of the *pfk* gene of *S. coelicolor* A3(2) and analysis of the deduced amino acid sequence (343 amino acids; 36,667 Da) revealed high similarities to the PP_i-PFK enzyme from *Amycolatopsis methanolica* (tetramer, nonallosteric; 70%) and to the allosteric ATP-PFK enzymes from other bacteria, e.g., *Escherichia coli* (tetramer; 37%) and *Bacillus stearothermophilus* (tetramer; 41%). Further structural and functional analysis of the two actinomycete PFK enzymes should elucidate the features of these proteins that determine substrate specificity (ATP versus PP_i) and allosteric (in)sensitivity.

In many organisms the glycolytic pathway is regulated at the level of the irreversible enzyme ATP-dependent phosphofructokinase (ATP-PFK) (EC 2.7.1.11) (14, 42). The most commonly encountered bacterial ATP-PFK is a tetramer of 35-kDa subunits, which is subject to allosteric inhibition by phosphoenolpyruvate (PEP) and activation by ADP and GDP. The ATP-PFKs from *Eucarya* and *Bacteria* show significant amino acid sequence similarity (3, 14, 17, 26, 28). A second type of ATP-PFK enzyme, found only in *Escherichia coli*, has a dimeric structure and is nonallosteric (24, 42).

An alternative type of phosphofructokinase exists that is dependent on inorganic pyrophosphate (PP_i) and has a more limited distribution (15, 29, 33). The PP_i-PFK enzymes (EC 2.7.1.90) isolated from *Bacteria* are usually dimeric and are not sensitive to regulation at the activity level (7, 29, 33). Full alignment of the ATP- and PP_i-PFK proteins reveals little similarity (15, 20, 25, 34). Interestingly, the actinomycete *Amycolatopsis methanolica* employs a tetrameric PP_i-PFK enzyme with characteristics between those of the ATP-PFKs and the PP_i-PFKs (2, 3). Its nonallosteric properties raised questions about the regulation of glucose metabolism in actinomycetes in general. This PP_i-PFK enzyme is also present in other actinomycetes, e.g., in members of the *Pseudonocardiaceae* (2) and *Actinoplanaceae* (38) and *Actinomyces naeslundii* (40). A search for PFK enzymes in other important genera of actinomycetes indicated the presence of an ATP-PFK in *Streptomyces coelicolor* A3(2) (2), genetically the most studied actinomycete with respect to antibiotic production and morphological differentiation (22). Relatively little information is available about primary metabolism and its regulation in this organism, which is a model for an important group of industrial bacteria (4, 5). Also, the molecular mechanisms of glucose repression of antibiotic biosynthesis in actinomycetes remain to be elucidated. Conceivably, characterization of regulatory steps in primary metabolism in *S. coelicolor* A3(2) and other actinomycetes will be important for further improvement of strains that overproduce secondary metabolites. Here we report the characterization of the ATP-PFK protein and the corresponding gene from *S. coelicolor* A3(2).

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MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used are listed in Table 1.

Media and growth conditions. *S. coelicolor* A3(2) strains were grown under standard conditions in YEME medium (19). *E. coli* strains were grown on Luria-Bertani medium at 37°C (37). When appropriate, 100 μg of ampicillin per ml and 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) were added. Agar (1.5% [wt/vol]) was added for solid media.

Preparation of extracts and enzyme assay. Cells were washed in buffer containing 50 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (pH 7.2), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 50 mM $(NH_4)_2SO_4$, and 0.1 mM phenylmethylsulfonyl fluoride (buffer A) and disrupted by passing three times through a French pressure cell at 140 MPa. Unbroken cells and debris were removed by centrifugation of the lysate at 40,000 × *g* for 30 min, and the supernatant used for the purification of the ATP-PFK. ATP-PFK (EC 2.7.1.11) activity (1 U = 1 μmol · min^{-1} · mg of $protein^{-1}$) was assayed in a reaction mixture with 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM KCl, 3 mM NH_4Cl, 5 mM dithiothreitol, 0.15 mM NADH, 10 mM fructose-6-phosphate (F-6-P), 0.9 U of fructose biphosphate aldolase, 5 U of triose phosphate isomerase, 0.85 U of α-glycerol-3-phosphate dehydrogenase, and limiting amounts of extract. The reaction was started by addition of 2.5 mM ATP. ATP solutions were adjusted to assay pH values before use. To obtain expression of *pfk*, *E. coli* DF1020 cells containing the appropriate plasmids were grown in Luria-Bertani medium at 37°C. At an A_{660} of 0.5, IPTG (1 mM) was added to induce transcription from the *lac* promoter, and growth was allowed to continue for 4 h. Cells were harvested by centrifugation at 7,000 × *g* for 15 min at 4°C and resuspended in 50 mM Tris-HCl (pH 7.5).

Purification of the ATP-PFK enzyme. All chromatographic steps were carried out in a System Prep 10 liquid chromatography system (Pharmacia LKB Biotechnology, Inc.) at room temperature. Fractions were immediately placed on ice.

Step 1: extract preparation. Extracts were prepared (see above) from YEME-grown cells (10 g [wet weight]) of *S. coelicolor* A3(2) strain MT 1109 harvested at the end of exponential growth (A_{430} = 1.5).

Step 2: protamine sulfate precipitation. A freshly prepared 10% (wt/vol) stock solution of protamine sulfate was added slowly with stirring to a final concentration of 0.2%. The mixture was centrifuged (40,000 × *g* for 15 min), and the supernatant was retained.

Step 3: ammonium sulfate fractionation. The solution was slowly adjusted to 40% saturation with solid $(NH_4)_2SO_4$ and centrifuged at 40,000 × *g* for 15 min. The supernatant was decanted, adjusted to 60% $(NH_4)_2SO_4$ saturation, and centrifuged again. The pellet was resuspended in a minimum volume of buffer A and dialyzed overnight against buffer A at 4°C.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics[a]	Source or reference
E. coli		
DH5α	*supE44ΔlacU169* (*ϕ80lacZΔM15*) *hsdR17 recA endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
DF1020	*pro*-82 *gln*V44(AS) λ$^-$ Δ*pfk*B201 *recA56 endA1* Δ(*rhaD-pfkA*)200 *thi-1 hsdR17*	*E. coli* Genetic Stock Center
S. coelicolor A3(2)		
MT 1109	Prototrophic, SCP1$^-$, SCP2$^-$	Gift from C. Smith
M145	Prototrophic, SCP1$^-$, SCP2$^-$	19
Plasmids		
6E10	Cosmid isolated from a library of *S. coelicolor* M145 DNA in *E. coli* cosmid vector Supercos-1	Gift from M. Redenbach
pST101	Ap^r, 2.7-kb *Pvu*II fragment in pBluescript KS$^+$ with *pfk* in the same orientation as the *lac* promoter	This study
pST401	Ap^r, 2.7-kb *Pvu*II fragment in pBluescript KS$^+$, with *pfk* in the opposite orientation to the *lac* promoter	This study
pAA101	Ap^r, 2.3-kb *Pvu*II fragment containing the *pfp* gene of *A. methanolica*	3
pBluescript KS$^+$	Ap^r, phagemid derived from pUC18 *lacZ*	Stratagene

[a] Ap^r, ampicillin resistant.

Step 4: anion-exchange chromatography. Protein from step 3 was applied to a Q-Sepharose column (HR10/10) previously equilibrated with buffer A (flow rate, 4 ml · min^{-1}). Bound proteins were eluted with a linear gradient of 50 to 200 mM $(NH_4)_2SO_4$ in buffer A. Fractions containing ATP-PFK activity were pooled and adjusted to 40% $(NH_4)_2SO_4$ saturation.

Step 5: hydrophobic interaction chromatography. Protein from step 4 was applied to a phenyl Superose column (HR5/5) equilibrated with buffer A containing 40% $(NH_4)_2SO_4$ saturation (flow rate, 0.5 ml · min^{-1}). Bound proteins were eluted with a decreasing linear gradient of 1.7 M to 50 mM $(NH_4)_2SO_4$ in buffer A. Fractions with ATP-PFK activity were pooled.

Step 6: gel filtration. Protein from step 5 was applied to a Superdex 200 column (XK 16/60) previously equilibrated with buffer A (flow rate, 1 ml · min^{-1}). Proteins were eluted with buffer A. Fractions containing ATP-PFK activity were pooled.

Step 7: anion-exchange chromatography. Protein from step 6 was applied to a Mono Q column equilibrated with buffer A (flow rate, 1 ml · min^{-1}). Bound proteins were eluted with a linear gradient of 0 to 0.6 M NaCl in buffer A. Fractions with ATP-PFK activity were pooled and diluted in an equal volume of buffer A with 80% $(NH_4)_2SO_4$ (1:1 dilution).

Step 8: hydrophobic interaction chromatography. Protein from step 7 was applied to an alkyl Superose column (HR 5/5) previously equilibrated with buffer A containing 1.7 M $(NH_4)_2SO_4$ (flow rate, 0.5 ml · min^{-1}). Bound proteins were eluted with a decreasing linear gradient of 1.7 M to 50 mM $(NH_4)_2SO_4$. Fractions with ATP-PFK activity were pooled; glycerol was added to a final concentration of 40% (vol/vol) before storage at −20°C.

Kinetic studies. Kinetic parameters were determined at 30°C and pH 7.5 and were calculated with Sigma Plot for Windows 2.0 (Jandell Scientific Software) by curve fitting with the Hill or Michaelis-Menten equation. Possible effectors of ATP-PFK were added separately (pH adjusted, 1 mM final concentration) to the assay mixtures with the purified enzyme, using near-K_m concentrations of the substrates F-6-P (1 mM) and ATP (0.5 mM).

Southern hybridizations. Chromosomal DNA from *S. coelicolor* A3(2), digested with the appropriate enzymes, was subjected to electrophoresis on a 0.8% (wt/vol) agarose gel and transferred to a nylon plus membrane (Qiagen, Basel, Switzerland) after alkaline denaturation (37). The membrane was probed at 65°C with a 1.7-kb *Bam*HI-*Eco*RV DNA fragment from pAA101 with the *A. methanolica pfk* (3) (Table 1). The DNA probe was made with the DIG DNA-labeling kit of Boehringer (Mannheim, Germany). The membrane was subsequently washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% (wt/vol) sodium dodecyl sulfate for 15 min and twice with 0.5× SSC plus 0.1% (wt/vol) SDS for 5 min.

Nucleotide sequencing. A nested set of unidirectional deletions of pST401 was constructed by using exonuclease III and mung bean nuclease, essentially as described by Henikoff (18). Double-stranded DNA was sequenced either by using the T7-Deaza kit (Pharmacia) according to the manufacturer's recommendations or by using the automated laser fluorescent DNA sequencer Vistra System with the labeled primer cycle sequencing kit (Amersham). The nucleotide sequence data were compiled and analyzed by programs supplied in the PC/Gene software package (Intelligenetics, Mountain View, Calif.).

Tree construction. The PFK alignment was made with Clustal W (41). The programs supplied in the PHYLIP 3.5c package were used to determine phylogenetic relationships (13); SEQBOOT was used to generate 100 data sets. Distance matrices were calculated with PROTDIS, using Dayhoff's PAM 001 matrix (12). A phylogenetic tree was subsequently constructed by the neighbor-joining method (35) implemented in the NEIGHBOR program (100 trees). A consensus tree was constructed with CONSENCE. Reliability of phylogenetic tree branches was tested by bootstrapping (13) using SEQBOOT.

Analytical methods. Estimation of molecular mass and protein concentrations, amino acid sequence analysis and alignments, and DNA manipulations were as described previously (2, 3).

Nucleotide sequence accession number. The nucleotide sequence presented in this paper was entered into GenBank under accession number U51728.

RESULTS

Purification of the ATP-PFK. ATP-PFK activity was readily detected in extracts of *S. coelicolor* A3(2) strain MT 1109. Use of a 50 mM TES (pH 7.2) buffer containing 5 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$ (or K_2SO_4 but not NH_4Cl), 5 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride (protease inhibitor) (buffer A) was essential for the retention of ATP-PFK activity throughout its purification to homogeneity (Table 2). Routinely, a 1,600-fold purification was achieved with a final yield of 17%. Only a single ATP-PFK was present, and no PP_i-PFK could be detected.

Properties of the ATP-PFK. Gel filtration of the pure, active enzyme revealed an M_r of 110,000 ± 10,000. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and immunoblots with polyclonal antibodies raised against the ATP-PFK protein PFKA of *E. coli* revealed a single band of 40,000 ± 5,000 Da for the *S. coelicolor* A3(2) ATP-PFK (data not shown). The enzyme displayed a broad temperature optimum, with at least 90% of activity retained between 30 and 50°C. The pH optimum for activity was between 7.5 and 8.0. Storage of purified enzyme at −20°C in buffer A with 40% glycerol did not result in significant loss of activity over a period of 1 month.

The ATP-PFK showed absolute specificity for its substrates ATP and F-6-P. Divalent cations were necessary for activity; Mg^{2+} (100%) could be replaced by other divalent ions, e.g., Mn^{2+} or Zn^{2+}, with a remaining activity of 67 or 40%, respectively. Addition of PEP (1 mM) reduced ATP-PFK activity to 33%. The steady-state PEP concentration in *S. coelicolor* A3(2) is not known. In other bacteria physiologically relevant PEP concentrations vary between 1 and 5 mM (31, 44).

Kinetics of the ATP-PFK. The ATP-PFK displayed Michaelis-Menten kinetics with respect to both substrates, reaching a V_{max} value of 165 U · mg^{-1} (Fig. 1). PEP strongly inhibited activity; the data for PEP could be better fitted with the Hill equation, giving Hill coefficients of 1.65 at 1 mM PEP (Fig. 1A) and 3.0

TABLE 2. Purification of ATP-PFK from YEME-grown cells of *S. coelicolor* A3(2)

Step	Amt of protein (mg)	Total activity (U)	Sp act (U · mg^{-1})	Purification (fold)	Yield (%)
1. Crude extract	1,243	107	0.086	1	100
2. Protamine sulfate	1,265	104	0.086	1	100
3. $(NH_4)_2SO_4$ fractionation	771	104	0.22	2.6	97
4. Q-Sepharose	106	72.4	0.683	7.9	68
5. Phenyl-Superose	8.8	78.4	8.91	103.6	73
6. Superdex 200	2.7	47	17.4	202.3	44
7. Mono Q	1.3	40	30.8	358	38
8. Alkyl-Superose	0.135	18.5	137	1,593	17

at 2.5 mM PEP (data not shown). PEP increased the substrate concentration at $0.5V_{max}$ ($S_{0.5}$) for F-6-P from 1.0 ± 0.07 to 2.3 ± 0.2 mM and decreased the V_{max} from 115 to 50 U · mg^{-1} (Fig. 1A). The affinity for ATP was calculated by using different F-6-P concentrations, giving a K_m for ATP that varied from 1.3 ± 0.13 to 0.4 ± 0.02 mM (Fig. 1B).

N-terminal amino acid sequence of the ATP-PFK. Twenty-nine of the first thirty-six N-terminal amino acids of the ATP-PFK were identified. Amino acids at positions 1 to 19 (except numbers 2, 3, 8, and 12) were assigned unambiguously. All other amino acids were tentatively assigned or remained unidentified (Fig. 2). The *S. coelicolor* A3(2) ATP-PFK N terminus has 50% identity with the PP_i-PFK from *A. methanolica* (3). Both actinomycete species possess DNA with a high G+C content and exhibit a marked bias in codon usage (46), providing an opportunity to use part of the gene encoding the PP_i-PFK protein as a probe for cloning of the *S. coelicolor* A3(2) *pfk*.

Screening of the *S. coelicolor* A3(2) strain M145 cosmid library. Southern analysis of total DNA of *S. coelicolor* A3(2) strain MT 1109 using part of pAA101, which contains the *pfp* gene of *A. methanolica* (Table 1), as a probe revealed a hybridizing *Pvu*II band of 2.7 kb. Two oligonucleotides designed to correspond to the N-terminal part of the PP_i-PFK of *A. methanolica* (3) hybridized with the same band (data not shown). We therefore concluded that the pAA101 probe specifically hybridized with the *pfk* of *S. coelicolor* A3(2). The same hybridization conditions and the pAA101 probe were used to screen the cosmid library of *S. coelicolor* A3(2) strain M145 DNA (32). One strongly hybridizing clone was identi-

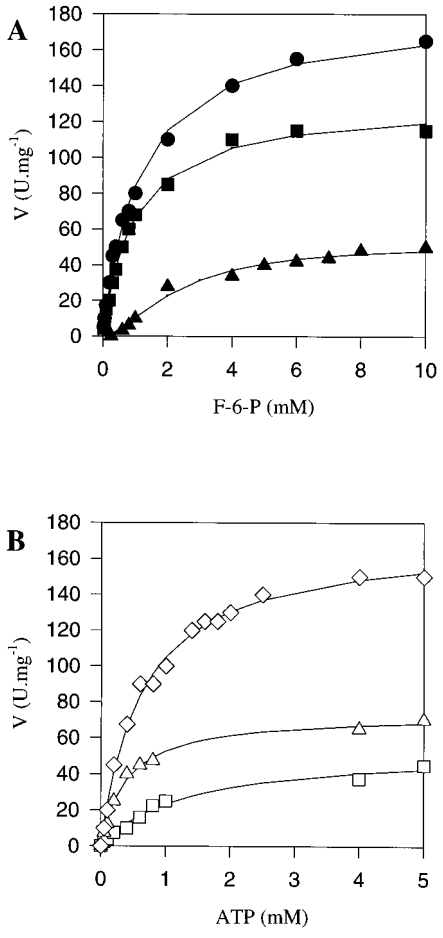

FIG. 1. (A) ATP-PFK activity versus concentration of F-6-P at different ATP and PEP concentrations. ●, 1 mM ATP; ■, 0.5 mM ATP; ▲, 0.5 mM ATP plus 1 mM PEP. (B) ATP-PFK activity versus ATP concentrations at 5 mM (◇), 1 mM (△), and 0.5 mM (□) F-6-P. All data were fitted to the Michaelis-Menten equation with the exception of the data with PEP, for which the Hill equation was used.

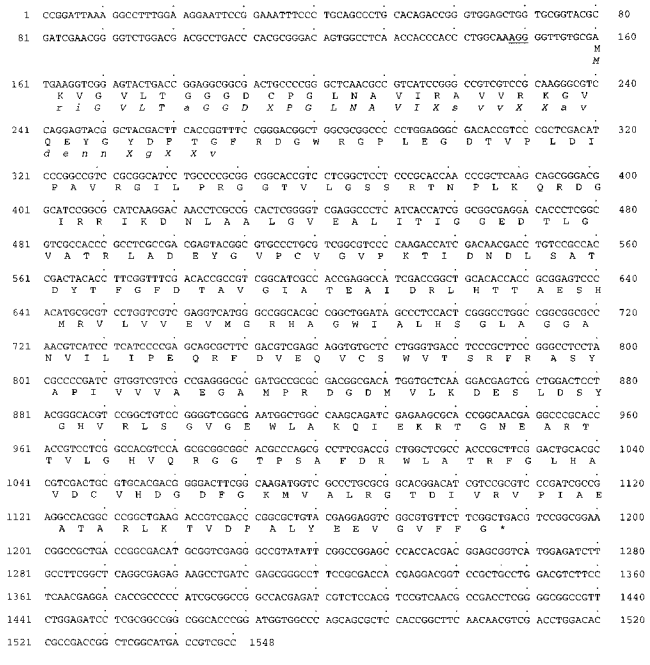

FIG. 2. Nucleotide sequence of part of the 2.7-kb *Pvu*II fragment containing *S. coelicolor* A3(2) *pfk*. A putative ribosome binding site preceding the *pfk* coding region is underlined. The predicted amino acid sequence is shown beneath the nucleotide sequence using the single-letter code. The N-terminal amino acid sequence (36 residues) determined for the ATP-PFK from *S. coelicolor* A3(2) is given in italics; lowercase characters indicate tentatively assigned amino acids. X, unidentified residue.

fied, corresponding to the cosmid 6E10 (Table 1). Southern analysis of a *Pvu*II digest of cosmid 6E10 revealed a 2.7-kb hybridizing fragment, which was subsequently cloned in pBluescript KS$^+$ in both orientations, yielding pST101 and pST401 (Table 1).

Heterologous expression of the *S. coelicolor* A3(2) *pfk* in *E. coli* DF1020. ATP-PFK activity was determined in extracts of *E. coli* DF1020 transformed with pST101 and pST401, before and after IPTG induction. This *E. coli* strain has a deletion in both the *pfk*A and *pfk*B genes (22); ATP-PFK activity in extracts therefore provides direct evidence for the presence and expression of the *pfk* of *S. coelicolor* A3(2). *E. coli* DF1020 cells harboring pST101, which contains *pfk* in the same orientation as the *lac* promoter, gave an ATP-PFK activity of 95 nmol · min^{-1} · mg of protein^{-1} after induction. *E. coli* cells harboring pST401, which contains *pfk* in the opposite orientation, had an activity of less than 1 nmol · min^{-1} · mg of protein^{-1}, as did cells harboring pBluescript KS$^+$. These results suggest that *pfk* is located entirely within the 2.7-kb *Pvu*II fragment and that its expression in *E. coli* is dependent on the *lac* promoter. The kinetic properties of the ATP-PFK enzyme expressed in *E. coli* matched those observed for the enzyme purified from *S. coelicolor* A3(2).

DNA sequence of the *S. coelicolor* A3(2) *pfk*. The nucleotide sequence of a 1.55-kb segment of the 2.7-kb *Pvu*II fragment was determined by double-strand sequencing. A single open reading frame of 343 codons, starting with an ATG codon preceded by a plausible ribosome binding site, was identified (Fig. 2). The deduced N-terminal amino acid sequence corresponds to the amino acid sequence determined experimentally for the purified enzyme, except for Lys-2, Val-3, and Gly-8, discrepancies that might reflect an observed high Arg background noise. The predicted molecular mass of ATP-PFK is 36,667 Da, which compares reasonably well with the estimated subunit size of the purified protein (40,000 ± 5,000 Da). The G+C content of *pfk* (71%) is consistent with that of other streptomycete genes. A search using BlastX (2) of the nonredundant nucleotide database at the National Center for Biotechnology Information Bethesda, Md., with the deduced amino acid sequence of PFK revealed extensive similarities to PFK proteins from various organisms.

Comparison of the *S. coelicolor* A3(2) ATP-PFK with other PFKs. An alignment of the ATP-PFK sequences from *S. coelicolor* A3(2) and other bacteria was made (Fig. 3). The PP$_i$-PFK from *A. methanolica* was also included since, of the PP$_i$-PFK enzymes, it shows the highest percent similarity to the ATP-PFKs (4). Interestingly, the ATP-PFK of *S. coelicolor* A3(2) is most similar to the PP$_i$-PFK from *A. methanolica* (70%) (Fig. 3). All other allosteric ATP-PFKs showed lower levels of similarity (*E. coli* 37%, *Bacillus stearothermophilus* 41%, *Thermus aquaticus* 44%) (Fig. 3).

Conservation of amino acid residues involved in substrate binding. Of the 11 amino acid residues involved in binding of F-6-P in the *E. coli* enzyme (39), 10 are conserved in *S. coelicolor* A3(2) PFK (Fig. 3). The exception is Arg-155 in the *E. coli* enzyme, which has been replaced by His-155 in the *S. coelicolor* A3(2) PFK. Of the 10 residues implicated in ATP binding in the *E. coli* enzyme (39), 2 are identical (Gly-12 and Arg-73), 4 represent a conserved substitution (Tyr-42 to Trp-41, Arg-78 to Lys-77, Asp-104 to Glu-103, and Ser-106 to Thr-105), and the final 4 are clearly different, with changes from Cys-74 to Thr-73, Gly-105 to Asp-104, Met-108 to Gly-107, and Gly-109 to Val-108.

Glu-187 in the allosteric PFKs of *E. coli*, *B. stearothermophilus*, and *T. aquaticus* is known to play an important role in the inhibition of PFK by PEP and in activation by ADP or GDP (6). A change to a neutral amino acid in the *E. coli* enzyme results in loss of activation by ADP and GDP and in altered binding of PEP. In the nonallosteric PFKs from *Spiroplasma citri*, *Lactobacillus delbrueckii*, and *Lactococcus lactis*, Glu-187 is replaced by Asp-187. In the *S. coelicolor* A3(2) enzyme and in the *A. methanolica* PP$_i$-PFK, Glu-187 is replaced by Asn-187, which may explain the insensitivity of these enzymes to activation by ADP or GDP. However, the *S. coelicolor* A3(2) enzyme is sensitive to inhibition by PEP, decreasing the affinity of the enzyme for the substrate F-6-P. In contrast, the *A. methanolica* PP$_i$-PFK is insensitive to each of these compounds (Fig. 3).

Phylogenetic relationships of PFKs. Previous studies of the PP$_i$-PFK of *A. methanolica* (3) suggested that ATP-PFKs and

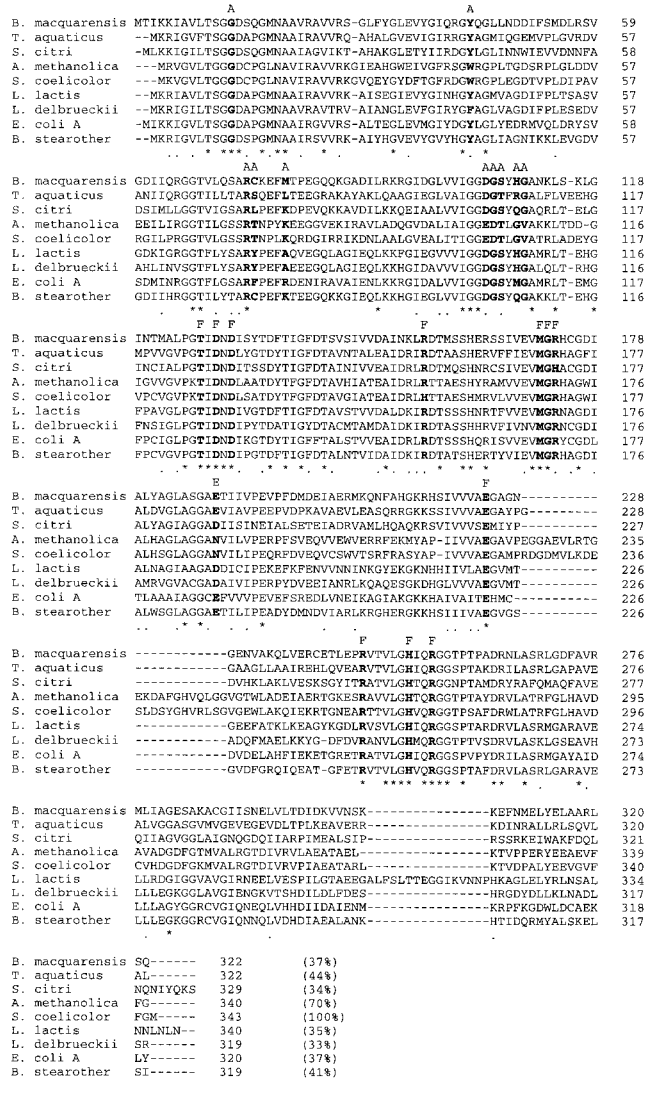

FIG. 3. Amino acid sequence alignments of the ATP-PFKs from *S. coelicolor* A3(2), *Bacillus macquarensis* (National Centre for Biotechnology Information accession number 433982), *B. stearothermophilus* (36), *L. lactis* (27), *L. delbrueckii* (8), *T. aquaticus* (47), *S. citri* (10), and *E. coli* (30) and the PP$_i$-PFK from *A. methanolica* (3). A, F, and E refer to residues implicated in binding ATP, F-6-P, and PEP, respectively, for the *E. coli* ATP-PFK. Alignment of the PFKs was made by using Clustal W (41). The percent similarity between the PFK from *S. coelicolor* A3(2) and each of the other PFKs is indicated in parentheses. *, identical residues; ., similar residues according to the following groupings: RK, NQ, DE, PAGST, VILM, FYW, H, and C.

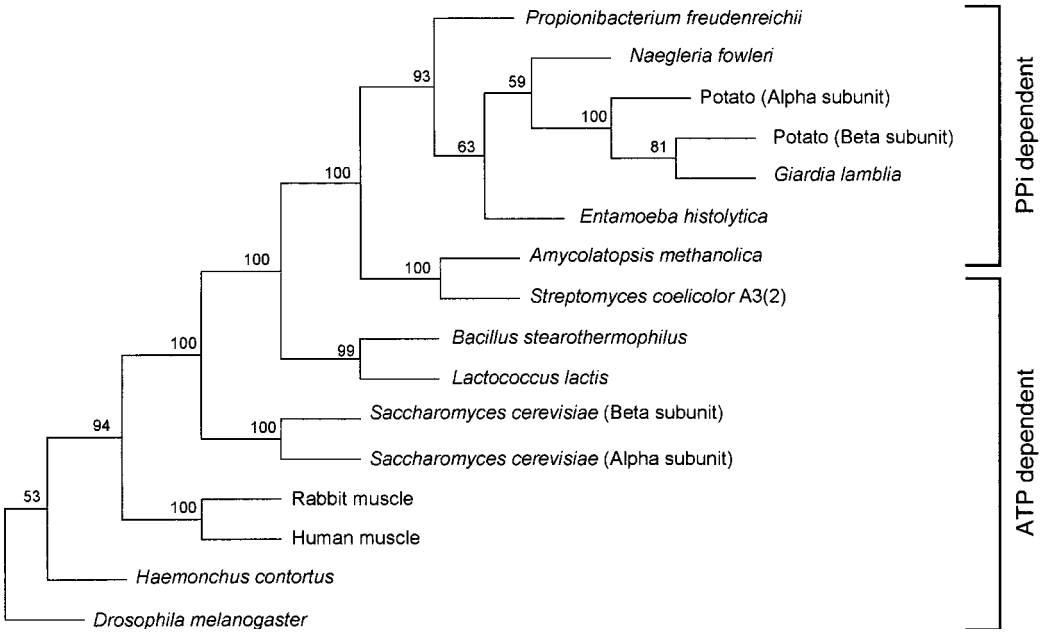

FIG. 4. Phylogenetic tree of PFKs. The tree is based on a distance analysis of the PFK segments involved in binding of ATP and F-6-P from *S. coelicolor* A3(2) (residues 1 to 284), *B. stearothermophilus* (residues 1 to 261) (36), *L. lactis* (residues 1 to 262) (27), *Saccharomyces cerevisiae* β subunit (residues 195 to 493), *Saccharomyces cerevisiae* α subunit (residues 204 to 500) (16), rabbit muscle (residues 14 to 310) (26), human muscle (residues 14 to 310) (28), *Haemonchus contortus* (residues 30 to 325) (23), *Drosophila melanogaster* (residues 16 to 313) (11), *A. methanolica* (residues 1 to 283) (3), *Entamoeba histolytica* (residues 34 to 298) (20), *Giardia lamblia* (residues 66 to 351) (34), potato α subunit (residues 85 to 369), potato β subunit (residues 39 to 322) (9), *Naegleria fowleri* (residues 16 to 287) (45), and *Propionibacterium freudenreichii* (residues 1 to 288) (25). A bootstrap value is indicated at each internal node (in percent) and is based on 100 data sets.

PP_i-PFKs constitute two different groups in the PFK family. The high overall similarity between the *S. coelicolor* A3(2) ATP-PFK, the *A. methanolica* PP_i-PFK, and other ATP-PFKs suggests that these proteins originated from a common ancestor. To test this hypothesis, a phylogenetic tree of PFKs was constructed with those parts of the full-length protein sequences that include the amino acid regions known to participate in substrate binding (3). The consensus tree (Fig. 4) shows that the PFKs from *S. coelicolor* A3(2) and *A. methanolica* form a separate cluster. This result is supported by the bootstrap values of 100%.

DISCUSSION

In this paper we report the first purification and characterization of an ATP-dependent PFK enzyme from an actinomycete, *S. coelicolor* A3(2) strain MT 1109. The data provide evidence that the glycolytic pathway of *S. coelicolor* A3(2) is regulated at the activity level. However, the in vivo importance of PFK in the overall control of carbon flux through the glycolytic pathway remains to be determined.

The *S. coelicolor* A3(2) ATP-PFK is most similar (70%) to the PP_i-PFK from another actinomycete, *A. methanolica*, although the two enzymes differ strongly in regulatory properties (2; this study). Residues involved in binding F-6-P in the *E. coli* and *B. stearothermophilus* ATP-PFKs are highly conserved in the *S. coelicolor* A3(2) enzyme. The degree of similarity between the *E. coli* and *S. coelicolor* A3(2) enzymes is lower in the ATP binding site. Interestingly, the same differences are observed in both the ATP-PFK of *S. coelicolor* A3(2) and the PP_i-PFK of *A. methanolica* (Fig. 3). It thus remains unclear what determines ATP and PP_i specificity in these actinomycete enzymes. Also, the structural features of the *S. coelicolor* A3(2) ATP-PFK and the *A. methanolica* PP_i-PFK that determine allosteric insensitivity remain to be elucidated.

It has been suggested that PP_i-PFKs evolved many times from ATP-PFKs (14). Our previous work on the phylogeny of the PP_i-PFK from *A. methanolica* showed clearly that this was not the case (3). PP_i-PFK enzymes apparently form a monophyletic group, and both ATP- and PP_i-dependent PFK enzymes probably evolved from a common ancestor. As described in this paper, the PFK enzymes from *S. coelicolor* A3(2) and *A. methanolica* form a separate cluster. Further work on the phylogenetic position of PP_i- and ATP-dependent PFK enzymes from other actinomycetes should clarify this interesting evolutionary question.

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